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Hemocyanin Subunit Composition and Oxygen Binding in Two Species of the Lobster Genus *Homarus* and Their Hybrids

CHARLOTTE P. MANGUM

Bodega Marine Laboratory, University of California, P. O. Box 247, Bodega Bay, California 94923[1]

Abstract. The monomeric subunit composition and O_2 binding properties of the hemocyanins (Hcs) of *Homarus americanus, H. gammarus* and their hybrids are very similar, though not identical. *H. americanus* Hc has six major electrophoretically separable polypeptide chains; *H. gammarus* Hc has four major and two minor chains; and the hybrid Hc has four major and one minor chain. Four chains co-migrate in all three groups, and the fifth chain in the hybrid co-migrates with a fifth chain in *H. gammarus*. Thus, qualitatively, the hybrid Hc is more like that of *H. gammarus* than *H. americanus*, a similarity reflected in respiratory properties. Although the O_2 affinity of the hybrid hemocyanin appears to lie intermediate between that of the two parent hemocyanins at 25°C, in fact it is significantly different from that of *H. americanus* but not *H. gammarus*. The cooperativity of the hybrid Hc also differs significantly from that of *H. americanus* but not *H. gammarus* Hc. The distinctive properties of *H. americanus* hemocyanin at 25°C are believed to be due to either or both of two chains: a unique and also invariant chain in *H. americanus*, and one that is present in *H. gammarus* and the hybrids but not in *H. americanus*. *H. americanus* Hc also appears to be slightly less sensitive to the allosteric modulator L-lactate. No difference in $CaCl_2$ sensitivity was found. At lower temperatures respiratory properties are indistinguishable. In adult *H. americanus* that had been held under identical conditions for long periods, variation in subunit pattern was not entirely absent, but it was smaller than that found in natural populations of other species. No differences in O_2 binding at 25°C were found in morphs differing qualitatively in one chain and quantitatively in two others. No effect of a combination of rearing temperature and diet was found on the Hc subunit composition of juveniles.

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Permanent address: [1]Department of Biology, College of William & Mary, Williamsburg, VA 23185-8795.

Introduction

The arthropod hemocyanins (Hcs) are multiples of hexamers built of 70–80 kDa polypeptide chains. Often the 2×6-mers predominate in the bloods of adult decapod crustaceans, including the lobster *Homarus americanus* (Olson *et al.*, 1988). The number of different monomers is usually large, with a dozen or more found in several species of *Uca* (Sullivan *et al.*, 1983; Callicott and Mangum, 1992; Mangum, 1992 and unpub. data). The monomers have been grouped into one of four categories on the basis of their electrophoretic mobilities, immunological reactions and roles in oligomer assembly (Markl, 1986). Within a species, the monomeric heterogeneity also plays a functional role in respiratory adaptation during the adult stage (Mason *et al.*, 1983; Mangum and Rainer, 1988; deFur *et al.*, 1990; Mangum *et al.*, 1991). By comparing morphs, the functional differences have been attributed to particular electrophoretic bands (Mangum and Rainer, 1988; Mangum *et al.*, 1991; Mangum, 1992).

The role of Hc subunit composition in bringing about functional differences between species is less clear. A survey of forty-two species of various degrees of taxonomic relatedness suggests a high degree of specificity (Reese and Mangum, 1992). More intensive investigation of the Hcs of seven species of the genus *Uca*, which are extremely polymorphic as well as heterogeneous, supports the inference of species specificity (Callicott and Mangum, 1992; Mangum, 1992; C. P. Mangum, unpub. data). In every case, including sibling species such as *U. panacea* and *pugilator*, even low frequency Hc morphs of a species can be readily distinguished from those of another. Functional

properties can also differ in sibling congeners with different latitudinal ranges. In comparisons of congeners that are less closely related, however, functional properties are more clearly related to environmental factors than to phylogenetic affinity or subunit composition (Reese and Mangum, 1992).

In the only two species in which the effect of laboratory acclimation has been examined, the subunit phenotype of an adult individual is not fixed (Mason *et al.*, 1983; deFur *et al.*, 1990; Callicott and Mangum, 1992). In *Callinectes sapidus*, moreover, the variation both in the laboratory and in nature can be related to environmental factors such as salinity and hypoxia (Mangum, 1990; Pihl *et al.*, 1991; C. P. Mangum and S. P. Baden, unpub. obs.). Thus the members of the highly polymorphic samples of natural populations may have been acclimated to different environmental (or nutritional) conditions.

Here I report data for the monomeric subunit composition and oxygen binding of the Hcs of the adults of two species of lobsters in the genus *Homarus* and the hybrid progeny of their spontaneous matings. The two parent species had been brought from their native Atlantic habitats to the Bodega Marine Laboratory, where they were held under identical conditions for periods far longer than the species investigated previously. They are known to be highly homozygous at 41 loci and, at most loci, the allozymic phenotypes of the two are either extremely similar or identical. It is believed that the two speciated allopatrically when isolated for the first time during the Pleistocene (Hedgecock *et al.*, 1977). In one species, I also examined the Hc subunit composition of juveniles which had been reared on either of two diets, and at different temperatures.

Materials and Methods

The sample

All available adults, a total of 36, were examined; they were large (28–42 cm from rostrum to tail), intermolt individuals. Two (one of each sex) belong to *Homarus gammarus* (Linnaeus), formerly known as *H. vulgaris;* they were collected near Iona, Scotland in 1975. They are the sole survivors of the larger sample characterized by Hedgecock *et al.* in 1977. Twenty-five adults (16 females, 9 males) are members of *Homarus americanus* H. Milne Edwards. All but one were caught on various dates in 1988–92 in waters surrounding Martha's Vineyard, Massachusetts, and had been held in the mariculture facility at the Bodega Marine Laboratory for periods ranging from three months (1 individual) to more than three years (3 individuals). One individual of *H. americanus* (age > 6 years) was born in the Bodega Marine Laboratory. Nine hybrid adults (7 fertile females, 2 infertile males) were progeny of spontaneous matings between *H. gammarus* and *americanus*. Most were produced in 1983 by a female *H. gammarus* and a male *H. americanus;* one, of unknown parentage, was born in 1978.

All adults had been fed the same diet of surf fish and shrimp, and had been held under identical photoperiods in the running seawater system of the Bodega Marine Laboratory. A seven year compilation of data (1985–91) indicates that the water temperature ranges from 10 to 15°C, and usually varies within about two degrees; over the four month period of sampling the salinity varied from 32.5–33.5‰, which is typical.

The Hc subunit composition of 14 juvenile *H. americanus* (8–10 cm length, both sexes), which had hatched in the Bodega Marine Laboratory 20–22 months earlier, was also examined. Half of these animals, which were their natural color, had been fed since stage IV a diet of brine shrimp, fish and crabs, and had been held at the seawater system temperature. The other half, phenotypic albinos, had been fed a diet based on casein; for the past year they had been held at room temperature (*ca.*, 23°C). The diets, rearing conditions and molt history of animals such as these were described in detail by Baum (1990).

Preparation of material and electrophoresis

Blood was taken from the base of the last leg and serum expressed from the clot in a tissue grinder. After centrifugation an aliquot of the material was dissociated to its monomers by dilution with 0.01 mol l^{-1} EDTA + 0.05 mol l^{-1} Tris (pH 8.9), to reduce light scattering; Hc concentration was estimated from the absorbance of dissociated material at 338 nm (Bausch & Lomb Spectronic 2000 spectrophotometer), using the extinction coefficient reported by Nickerson and van Holde (1971). An additional aliquot was diluted (1:10 or 1:30, depending on concentration) with the dissociating buffer for electrophoresis, and the remainder frozen for future use. Absorbance of the material from several individuals, detailed below, was compared at 280 and 338 nm.

PAGE electrophoresis of native monomers was carried out at constant current according to Hames and Rickwood (1985). Following determination of the Hc phenotype in each individual, the variants among *H. americanus* were examined several times in adjacent lanes on the same gels. Representatives of each of the three groups were also compared many times on the same gels. Finally, gels were overloaded with six times the usual amount of material, the presence of Cu was determined according to Bruyninckx *et al.* (1978), and then the gels were stained as usual with Coomassie Blue.

Oxygen binding

On the basis of the PAGE, particular individuals were selected for a second bleeding, performed within a week

of the final phenotype determination. Serum was dialyzed overnight, against seawater for most of the measurements or against a Tris malcate buffered salt for the experiments on inorganic ion sensitivity. Oxygen binding was determined within a few days, using the cell respiration method (Mangum and Lykkeboe, 1979).

Data analysis

Bohr plots of the values for P_{50} (oxygen affinity) were described by regression lines and their 95% confidence intervals compared. Mean values for n_{50} (cooperativity) and Hc concentration were compared by Student's *t*-test. The data for O_2 binding as a function of [$CaCl_2$] were analyzed similarly. However, the nonlinearity of the response of P_{50} to [NaCl] and [Na_2SO_4] precluded statistical analysis.

Results

Hemocyanin concentration

Adults of *Homarus americanus* had significantly ($P = .02$) higher levels of Hc [6.11 (±0.41 S.E.) g 100ml^{-1}] than the hybrids [4.18 (±0.70 S.E.) g 100 ml^{-1}]. The values for the two members of *H. gammarus* (2.75 and 4.84 g 100 ml^{-1}) also fall below the 95% confidence interval around the mean of the *H. americanus* sample. In the *H. americanus* data there is no clear trend with length of time in the laboratory, suggesting that the nutritional state of the animals was good. The juveniles of this species had considerably lower Hc concentrations [1.02 (±0.15) g 100 ml^{-1}], which were unrelated to diet.

Monomeric subunit composition

The two adult *Homarus gammarus* had identical Hc phenotypes, which were also the same as that of one of the two individuals examined two years earlier (C. P. Mangum, unpub. obs.). Four high density (or major) and two intermediate density (or minor) electrophoretic bands separated by charge (Fig. 1). All six were positive for Cu.

The 25 adults of *H. americanus* exhibited very similar but not identical Hc phenotypes (Fig. 1). As many as eight bands separated on the lower third of the gels, four of which had co-migrants in *H. gammarus* (Fig. 1). The two most anodic bands (1 and 2) were always present in trace quantities, if at all. Material at their position appeared to quench the fluorescence of bathocuproine sulfonate, indicating the presence of Cu. However, it was not possible to ascertain the site of the quenching more precisely; only one of the two may contain Cu. These bands had no co-migrants in *H. gammarus* or the hybrids.

In *H. americanus* bands 3–8 could reach high concentrations. The gels on which the best separation was obtained exhibited less density in the middle of the material designated as bands 3 and 4, suggesting the presence of two chains that are similar in charge and extremely difficult to resolve. Moreover, the leading edge of this material clearly co-migrated with bands 2 in the hybrid and 3 in *H. gammarus*, whereas the trailing edge clearly lagged behind. Thus I assigned two numbers (3 and 4) to this position of the *H. americanus* material, even though the separation was not great enough to photograph. In addition, I was not able to decide whether the trailing material was present in all 25 individuals. The quantities of chains 6 and 7 in *H. americanus* are similar to the corresponding ones in *H. gammarus*, but chains 4 and 8 always occurred in higher levels in *H. americanus* than *H. gammarus* (Fig. 1).

In the early PAGE, band 5 in *H. americanus* did not appear to be sharply delineated at its leading and trailing edges. It was the only high density band that clearly varied qualitatively as well as quantitatively, ranging from absent (3 adults) to low concentration (5) to high concentration (17). Since I suspected that this band might not be a Hc chain, I examined the ratio of the absorbance at the protein peak (280 nm) and the active site (338 nm). According to this index, however, the total Cu content of *H. americanus* samples containing maximal levels of band 5 did not clearly differ from those that lacked it; nor did it differ from the samples from *H. gammarus* and the hybrids, none of which contained co-migratory material. In addition, on subsequent gels band 5 was as sharply delineated as the rest (Fig. 1).

Bands 6 and 8 varied quantitatively, though the magnitude was not great. They decreased concomitantly to intermediate levels in two of the 25 animals; band 8 was intermediate in two additional individuals in which band 6 remained maximal. Band 7 appeared to be absent in a single individual in which 6 and 8 were maximal. This female, from which larvae had hatched two months earlier, had been in the laboratory for only three months. All other individuals had maximal levels of these three chains.

Although I did not investigate material held at temperatures above freezing, there was no correlation between phenotype and age of frozen preparations; this has been true in my experience with Hcs from all species examined thus far. In the present case the same banding pattern was observed before and after four months. Finally, material prepared on a second occasion from four of the same individuals three months after the first bleeding showed no change in phenotype.

The hybrids, all adults, exhibited a single phenotype which did not vary quantitatively or qualitatively. With the exception of the higher levels of band 2, it resembles the phenotype of *H. gammarus* more than that of *H. americanus* (Fig. 1). The hybrid Hc has four major chains and one minor chain, all of which correspond in mobility

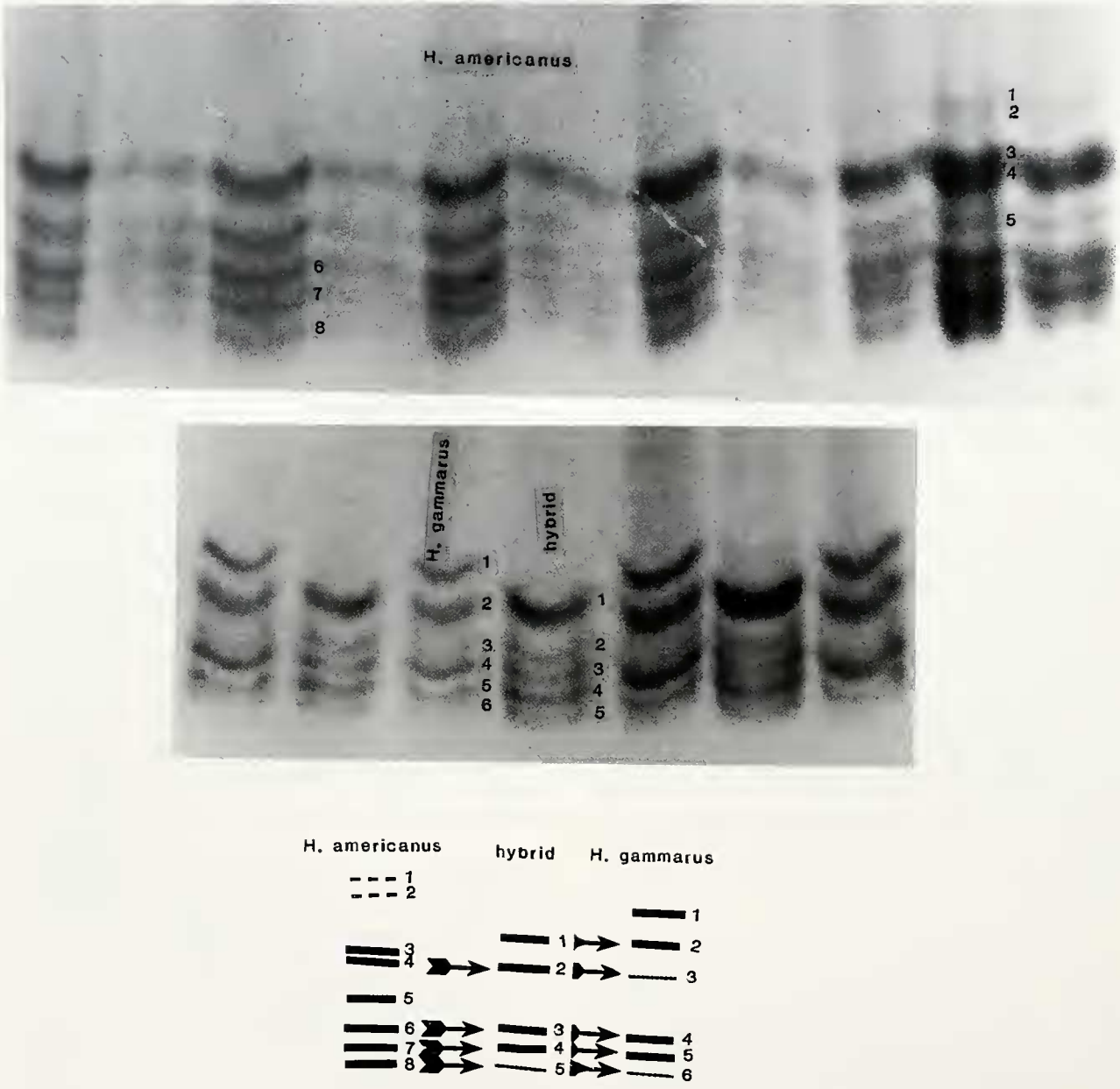

Figure 1. Banding patterns of the dissociated hemocyanins of the two parent species of *Homarus* and their hybrids, and a diagram illustrating the correspondence (arrows) of band positions. The anode is at the top. In the gel for *H. americanus,* each pair of lanes shows a sample from a different individual in higher (left) and lower (right) concentrations. The lanes on the far right were overloaded to show the anodic material (numbered 1 and 2) that occurs in trace quantities. The cathodic triplet of bands in this species is more clearly shown in lanes that were not overloaded. The middle panel shows *H. gammarus* (1 individual) and the hybrid Hc (1 individual) in alternating lanes.

to one of the six chains of *H. gammarus.* The hybrids differ from both parents, but from *H. gammarus* only in the absence of the most anodic band (*H. gammarus* band 1) and the higher levels of hybrid band 2. They differ from *H. americanus* in the absence of its three most anodic bands (*H. americanus* bands 1–3), in the absence of *H. americanus* band 5, in the consistently lower quantity of their most cathodic chain (hybrid band 5) and in the presence of a distinctive band 1.

As in all other species I have examined (*e.g.,* Mangum *et al.,* 1985; Mangum, 1992), the phenotypes of males and females in each of the three groups were indistinguishable—no sex specific material was present.

The juvenile *H. americanus* were indistinguishable from the adults. Six of the seven members of each dietary-thermal group had the maximum number of bands. One in each group lacked chain 5. Chains 6–8 were invariably present in maximal concentrations.

Oxygen binding

First, the intraspecific variation in *H. americanus* was examined. One adult lacked band 5 and also had minimal (=intermediate) levels of chains 6 and 8; at 25°C, however, the oxygen binding properties of its Hc (stripped of organic co-factors) were indistinguishable from those of another individual containing maximal amounts of all eight bands. Therefore the data have been combined for presentation. The coefficient of determination (r^2) for the regression line describing P_{50} in Figure 2 is 0.962, further affirming the absence of a perceptible effect of phenotype. The single individual with low quantities of band 7 had been sacrificed at the time the O_2 binding measurements were performed.

Second, the Hcs of the two parent species were compared. At all but the lowest pH investigated, Hc O_2 affinity at 25°C is significantly lower in *H. gammarus* than *H. americanus*, though the difference is fairly small (Fig. 2). Third, the hybrid Hc was compared with each of the parent Hcs. Whereas the data for the hybrid Hc appear to be intermediate between those for the two parent species, the difference from *H. gammarus* is not significant even in the middle of the pH range investigated. In contrast, the difference between the hybrid and *H. americanus* is significant throughout most of the pH range examined (>7.2).

The mean value for cooperativity is somewhat smaller (P = .001) in *H. americanus* (3.24 ± .11 S.E.) than *H. gammarus* and the hybrids (3.95 ± .13), which do not differ from one another (P = .15). Thus the respiratory properties of the hybrid Hc are also more like those of *H. gammarus* than *H. americanus*.

At lower temperatures, the significant differences disappear completely. Ninety-five percent confidence intervals around regression lines fit to the O_2 affinity data in Figure 3 overlap fully throughout the pH range investigated. Often this is true because the numerical values diminish and are thus more difficult to distinguish, but in this example there is not even an apparent trend. Mean values for cooperativity do not differ (P = .2–.8). As a result, *H. americanus* Hc is less temperature sensitive than the other two Hcs, though only in the 15–25°C range. For that range, the apparent heat of oxygenation (ΔH) is only −2.4 kcal mol^{-1} for *H. americanus* Hc (pH 7.6), whereas the value for the hybrid Hc is −5.6, and the value for *H. gammarus* Hc is −6.6. For the range 5–15°C the value of ΔH for all three Hcs is −9.4 kcal mol^{-1} (same pH).

H. americanus Hc is slightly less sensitive to the allosteric effector L-lactate (Fig. 4) than *H. gammarus* Hc; once again, the sensitivity of the hybrid Hc appears to be intermediate. At pH 7.6 the addition of 10 mmol l^{-1} lactate changes log P_{50} of *H. americanus* Hc by 0.166, *H. gammarus* Hc by 0.232, and the hybrid Hc by 0.203. However, O_2 affinity in *H. gammarus* and the hybrids is

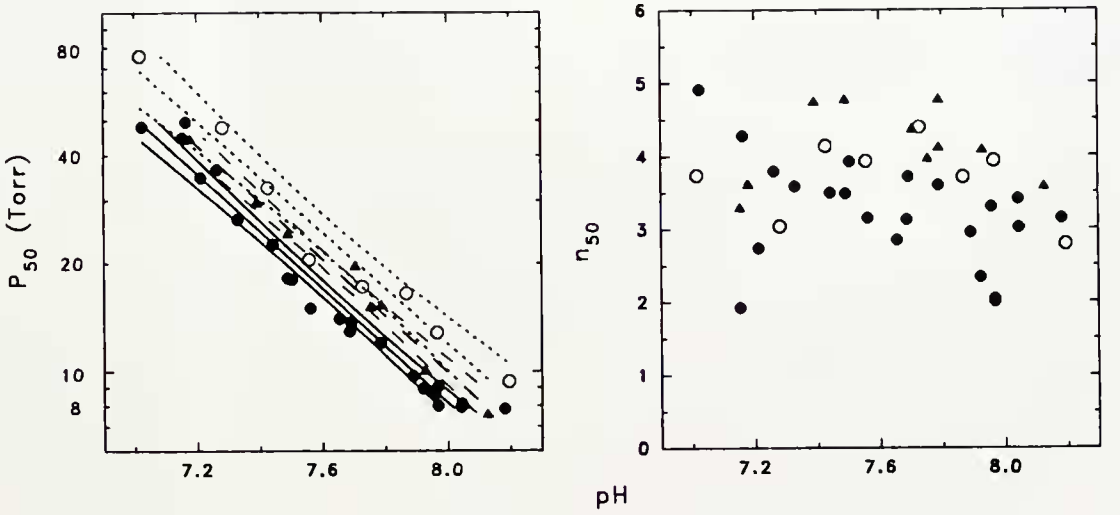

Figure 2. Oxygen binding at 25°C of *Homarus americanus* (closed circles, solid lines), *H. gammarus* (open circles, dotted lines) and their hybrid (triangles, dashed lines) hemocyanins. The curves are fitted regression lines ± 95% confidence intervals. 0.05 mol l^{-1} Tris maleate buffered seawater. Material obtained from two individuals of *H. americanus* was used (see text), whereas *H. gammarus* and the hybrids are represented by a single individual.

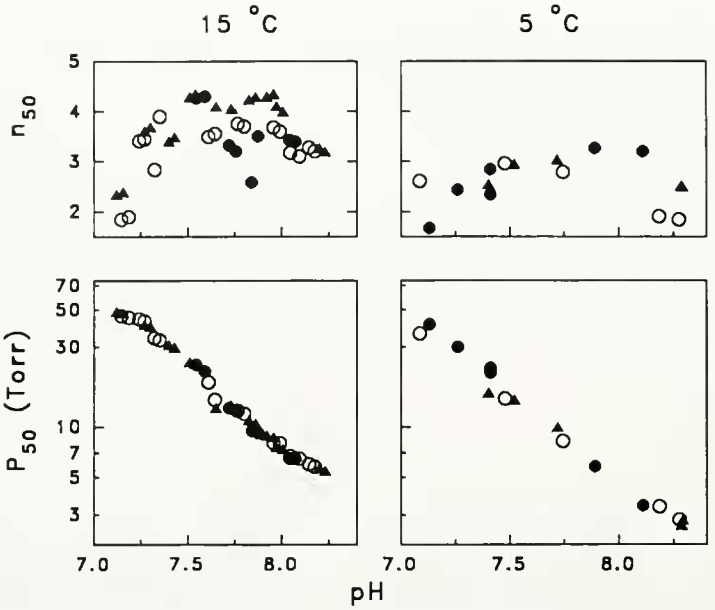

Figure 3. Oxygen binding at 15 and 5°C of *H. americanus* (closed circles), *H. gammarus* (open circles) and their hybrid (triangles) hemocyanins. The regression lines and confidence intervals were omitted for clarity. 0.05 mol l^{-1} Tris maleate buffered seawater. Origin of material as in Figure 2.

not quite significantly different in the presence of lactate, even in the middle of the pH range. In the presence of lactate, O_2 affinity of *H. gammarus* Hc remains significantly lower than that of *H. americanus* throughout the pH range investigated. In contrast, the hybrid Hc has a significantly lower O_2 affinity than that of *H. americanus* Hc only at high pH. In all three groups cooperativity is significantly diminished in the presence of lactate. The mean value drops from 3.2 to 2.86 ± .05 S.E. (P = .05) for *H. americanus* Hc, from almost 4 to 3.06 ± .24 (P = .05) for *H. gammarus* Hc, and from almost 4 to 2.95 ± .29 (P = .002) for the hybrid Hc.

The sensitivity of the three Hcs to $CaCl_2$ is indistinguishable (Fig. 5). Regression lines and their 95% confidence intervals overlap fully throughout the concentration range investigated. Mean values for cooperativity do not differ (P = .50–.75).

NaCl clearly raises Hc O_2 affinity and lowers cooperativity of *H. americanus* Hc (Fig. 5). Once again, however, the different morphs were indistinguishable, and the data were combined for presentation. In contrast to the allosteric effect of Ca^{2+}, the relationship between P_{50} and NaCl is nonlinear on logarithmic coordinates. I used high concentrations of Na_2SO_4, prepared from the decahydrate,

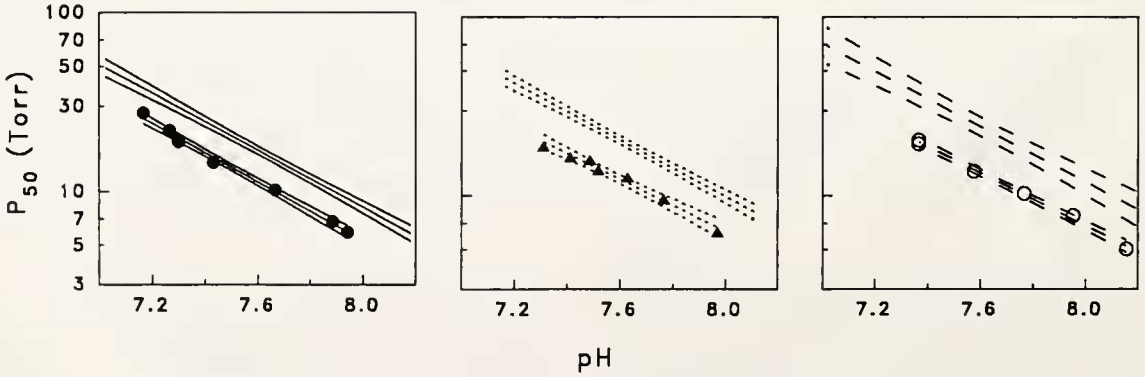

Figure 4. Lactate sensitivity of *H. americanus* (left panel: closed circles, solid regression lines ± 95% confidence intervals), *H. gammarus* (right panel: open circles, dotted lines) and their hybrid (middle panel: triangles, dashed lines) hemocyanins. Origin of material as in Figure 2. Control curves reproduced in each panel from Figure 2, 25°C, 0.05 mol l^{-1} Tris maleate buffered seawater.

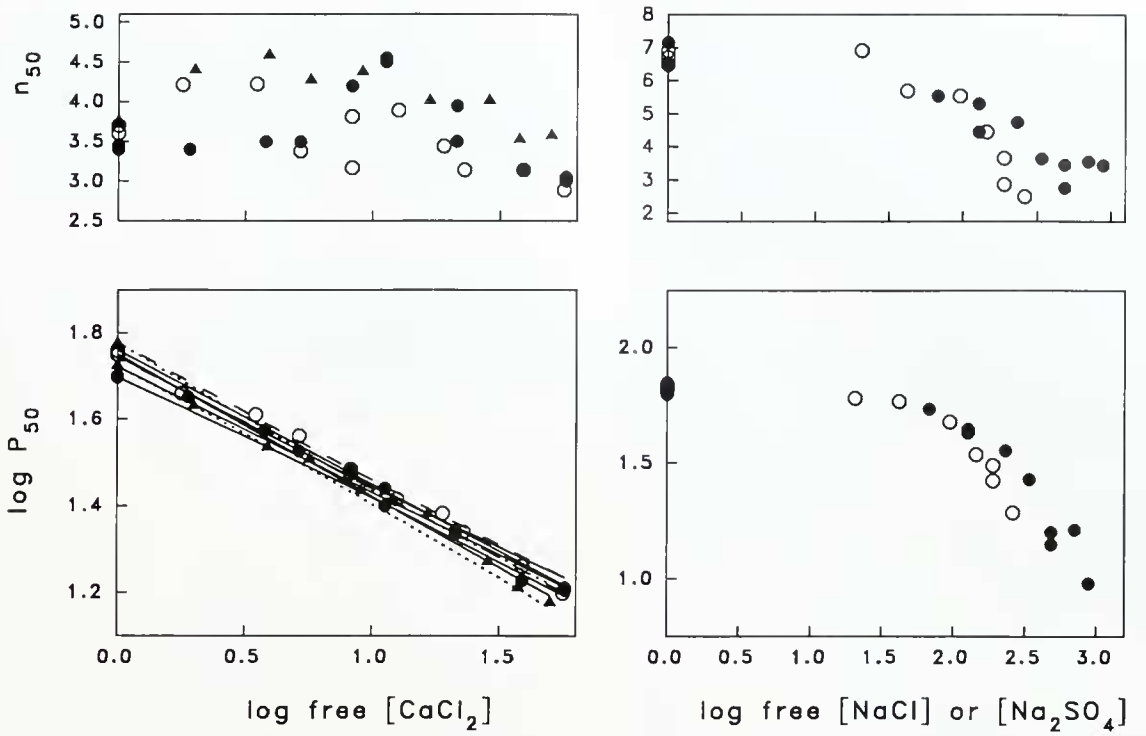

Figure 5. Inorganic ion sensitivities of lobster hemocyanins. The units of free ion concentrations (antilogs) are mmol l^{-1}. Left panels: *H. americanus* (closed circles, solid lines), *H. gammarus* (open circles, dashes) and their hybrids (triangles, dots). 0.05 mol l^{-1} Tris maleate buffer (pH 7.7) + 0.1 mol l^{-1} NaCl. Right panels: the response of *H. americanus* Hc to NaCl (closed circles) and Na_2SO_4 (open circles). 0.05 mol l^{-1} Tris maleate + 0.01 mol l^{-1} $CaCl_2$, 25°C. Origin of material as in Figure 2.

to examine specificity. The response differed very little from that of NaCl (Fig. 5), and the apparent difference may lie within the error of preparing an accurate solution of a highly hydrated salt (especially at a marine laboratory).

Discussion

The essentially non-specific sensitivity of *Homarus americanus* Hc to NaCl is further evidence that the inorganic ion responses of the crustacean Hcs are not all alike. The response of this Hc differs from that of portunid crab Hcs (Truchot, 1975; Mason *et al.*, 1983), which are insensitive to NaCl, but resembles that of penaid shrimp Hcs (Brouwer *et al.*, 1978; Mangum and Burnett, 1985). From a physiological point of view, however, NaCl sensitivity is unlikely to be important in *H. americanus*, a stenohaline species.

According to Hedgecock *et al.* (1977 and pers. comm.), the genetic distance between the two parent species of *Homarus*, though significant, is so small that the numerical value is closer to expectation for subspecies than species. Thus it is of particular interest that the present findings support the inference of species specificity of Hc subunit composition (Reese and Mangum, 1992). Although *H. gammarus* is monomorphic for the common *H. americanus* allele at 30 allozymic loci, neither of the two parent Hcs in the present sample could be confused with the other. As in the sibling species of *Uca* (Mangum, 1992 and unpub. obs.), this inference is true in spite of intraspecific variation. In *H. americanus*, band 3 is both diagnostic of the species and, at least in the present sample, invariant. Material that co-migrates with chains 1 and 2 of *H. gammarus* is clearly absent from *H. americanus*. Furthermore, the hybrid Hc is structurally distinct from either parent.

The present findings also support the inference of little interspecific genetic distance. Even though they are not identical, the two parent Hcs are more similar than any of the *ca.* 50 Hcs we have examined thus far, with the exception of *Menippe adina* and *M. mercenaria* Hcs (Reese 1989). Like the lobsters, these two sibling species of stone crabs are also believed to have speciated recently, and they also hybridize spontaneously (Bert, 1986).

In both structural and functional properties the hybrid Hc resembles that of one parent more than the other. It has only one less chain than *H. gammarus* Hc but several fewer than *H. americanus* Hc. The electrophoretic be-

havior of each of the five hybrid chains is identical to that of some one of the *H. gammarus* chains, whereas hybrid chain 1 has no co-migrant in *H. americanus*. These relationships are reflected in the O_2 binding of the Hcs in a complete saline, though only at high temperature.

In stage IV through adult *H. americanus*, SDS PAGE separates three Hc chains (Olson *et al.*, 1988; Olson and McDowell, 1989). As is often the case (*e.g.*, Sullivan *et al.*, 1983), additional bands are revealed when the separation is made by charge.

In neither juveniles nor adults of this species can the Hc be categorized as strictly monomorphic at the level of quaternary structure, despite prolonged acclimation of the donors. Moreover, in juveniles the variation is distinctive of neither the stage nor the thermal-nutritional history. In both stages, however, the variation is much smaller than in samples of natural populations of several species of brachyuran crabs (Mangum, 1990, 1992; Callicott and Mangum, 1992). This generalization is true of respiratory properties of the adults as well. Although the sample size is much smaller in the present investigation, the inference remains unchanged when the comparison is made with, for example, the 14–20 individuals of *Callinectes sapidus* investigated by Mangum *et al.* in 1991.

I emphasize that the small amount of Hc variation found here may not accurately represent natural populations of *H. americanus* (much less *H. gammarus*). Nor is it clear that the lack of variation results from prolonged acclimation rather than limited genetic diversity, as found at other loci (Tracey *et al.*, 1975; Hedgecock *et al.*, 1977). However, I note that the inference of allozymic similarity in the species was made from samples of populations on either side of Cape Cod but not Cape Hatteras, the greater geographic barrier (*e.g.*, Friedrich, 1973; National Geographic Society, 1985).

The present findings suggest that, given the common acclimation, the differences observed both within *H. americanus*, and between this species and the other two groups, represent a fixed condition in an adult individual. Although only intermolt animals were investigated here, the finding of no change with molt stage in *Callinectes sapidus* (Mangum *et al.*, 1985) has recently been confirmed in *H. americanus* (N. B. Terwilliger, pers. comm.). More important in the present context, the virtual identity of most respiratory properties of the three Hcs appears to reflect the notable similarity of the electrophoretic phenotypes. Conversely, it is reasonable to suggest that the slightly higher O_2 affinity and lower cooperativity of *H. americanus* Hc at high temperature are due to the chains that are unique to one of the three groups. Perhaps the most likely candidate is chain 2 in *H. gammarus* (=1 in the hybrids), which is absent in *H. americanus*. However, the possibility that band 3 is invariant as well as unique to *H. americanus* cannot be excluded. Bands 1 and 2 are never present in *H. americanus* in more than trace quantities, and morphs containing or lacking chain 5 did not differ in O_2 binding. The latter inference would be unwarranted only if the effect of chain 5 was exactly compensated by an equal and opposite effect of chains 6 and 8, which were also variables in the comparison.

Acknowledgments

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